THE FORMATION OF SUBNUCLEAR AGGREGATES IN NORMAL AND SYNCHRONIZED PROTOZOAN CELLS[1]

OTTO H. SCHERBAUM, ALLAN L. LOUDERBACK AND THEODORE L. JAHN

Department of Zoology, University of California, Los Angeles 24, Cal.

Since Bütschli (1876) established the nuclear dualism in ciliates, there has been much speculation about the biological role of the micro- and macronucleus. The mitotic behavior of the micronucleus, with its delicate apparatus for chromosomal segregation, led to the generally accepted view of the importance of the micronucleus in inheritance and reproduction. The macronucleus, on the other hand, which was found to divide "simply" by pinching in two, was considered to be concerned "only" with the regulation of metabolic functions in the cell.

This concept of the duality of nuclear function as formulated by Hertwig in 1889, was substantiated by Goldschmidt (1904) and Popoff (1908). These authors distinguished between the genetically-active idiochromatin and the trophochromatin, which was concerned exclusively with the cellular metabolism. In the uninuclear protists both types of chromatin were considered to be present in one nucleus, while in ciliates the idiochromatin was confined to the micronucleus and the trophochromatin was found in the macronucleus only.

The view of the dualistic function of the nuclei in ciliates was abandoned after experimental data accumulated showing the controlling role the macronucleus plays in the processes of cell division and regeneration (Grell, 1950). The genetic importance of the macronucleus in ciliates stimulated cytological studies of its structure. A considerable body of evidence has been accumulated during the past 30 years showing Feulgen-positive bodies in the cytoplasm, which could not be accounted for by "macronuclear fragmentation," the process of disintegration of the macronucleus upon conjugation of two cells. These bodies often have a spherical shape, and after Feulgen staining show the homogeneous appearance of micronuclei. Very often these bodies have been erroneously described as micronuclei, a fact which was pointed out by Kidder (1933). Diller (1936) observed simple fragmentations of the macronucleus in *Paramecium aurelia*, and he used the term "hemixis" to denote such autonomous changes of the macronucleus which are not related to sexual phenomena or binary fission.

It is believed now that the macronucleus of the ciliates consists of many diploid subnuclei (Sonneborn, 1947). We therefore propose the term "subnuclear aggregates" (SNA's) for the Feulgen-positive material lost or expelled from the macronucleus into the cytoplasm. The formation of SNA's may occur 1) by simple extrusion of Feulgen-positive material from the macronucleus, or 2) by loss during the process of binary fission of the macronucleus. An example of extrusion of chromatin masses from the macronuclear anlagen in the exconjugates of *Ancistruma isseli* was described by Kidder (1933), and a spontaneous "budding" of

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macronuclei, independent of cell division, in *Ichthyophthirius multifiliis* was found by Haas (1933). In the course of binary fission of the macronucleus of *Colpidium colpoda* Kidder and Diller (1934) described how some of the nuclear material is left behind in the fission plane. This material becomes condensed and finally disappears. A similar phenomenon was described by Furgason (1940) in the amicronucleate strain T of *Tetrahymena pyriformis* and by McDonald (1958) in *Tetrahymena pyriformis* H.

These frequently observed chromatin extrusions from the macronucleus led Kidder and Diller (1934) to the suggestion of a presumptive role for this phenomenon. It was thought extrusion might be a manifestation of a universal principle of nuclear reorganization which, in turn, could account for a high division rate. However, no quantitative studies have so far been carried out on the formation of the SNA's, their frequency of formation, and their absolute size in various phases of population growth.

The system of synchronous cell division in *Tetrahymena pyriformis*, strain GL, as worked out by Scherbaum and Zeuthen (1953, 1955), was used for the study of this phenomenon. During the induced synchrony about 85 per cent of the cells are in the visible stage of fission and all stages of SNA formation can readily be found.

Method

The amicronucleate strain GL of *Tetrahymena pyriformis* was grown principally as described earlier (Scherbaum and Zeuthen, 1955). The growth medium was two per cent proteose peptone (Difco) with 0.5 per cent glucose and 0.1 per cent liver fraction L (Wilson Laboratories) in glass-distilled water. Salts were added as in the basal medium A of Kidder and Dewey, except that phosphates were omitted. The medium was filtered and autoclaved at 15 pounds for 15 minutes. One ml. of a three-day-old stock culture (approximately 2×10^5 cells per ml.) was used for the inoculation of 150 ml. of culture medium in a 500-ml. culture flask. The flask was submerged in a temperature-controlled water bath, which was mounted on a shaker.

Samples of 5 ml. were removed from the experimental flask at regular intervals for counting (Scherbaum, 1957) and for nuclear preparations. For the latter, the samples were concentrated by centrifugation in a hand centrifuge and the supernatant removed by suction. The concentrated cell suspension was fixed in one per cent aqueous osmic acid for two minutes. The cells were removed from the fixative by centrifugation, washed in water, and passed through alcohol (30 per cent to 100 per cent). The cells were then pipetted onto albuminized coverslips, slightly dried to affix the cells to the glass surface, and transferred to absolute alcohol for ten minutes. The coverslips were stored in 70 per cent alcohol. For the Feulgen reaction the samples were hydrolyzed in 1 N HCl at 60° C. for 12 minutes and exposed to the Schiff reagent for one hour.

Results

At an approximate population density of 5×10^3 cells per ml. the first sample was removed. This served as the control for normal exponential multiplication. The second sample was removed during the synchronous division. For the induc-

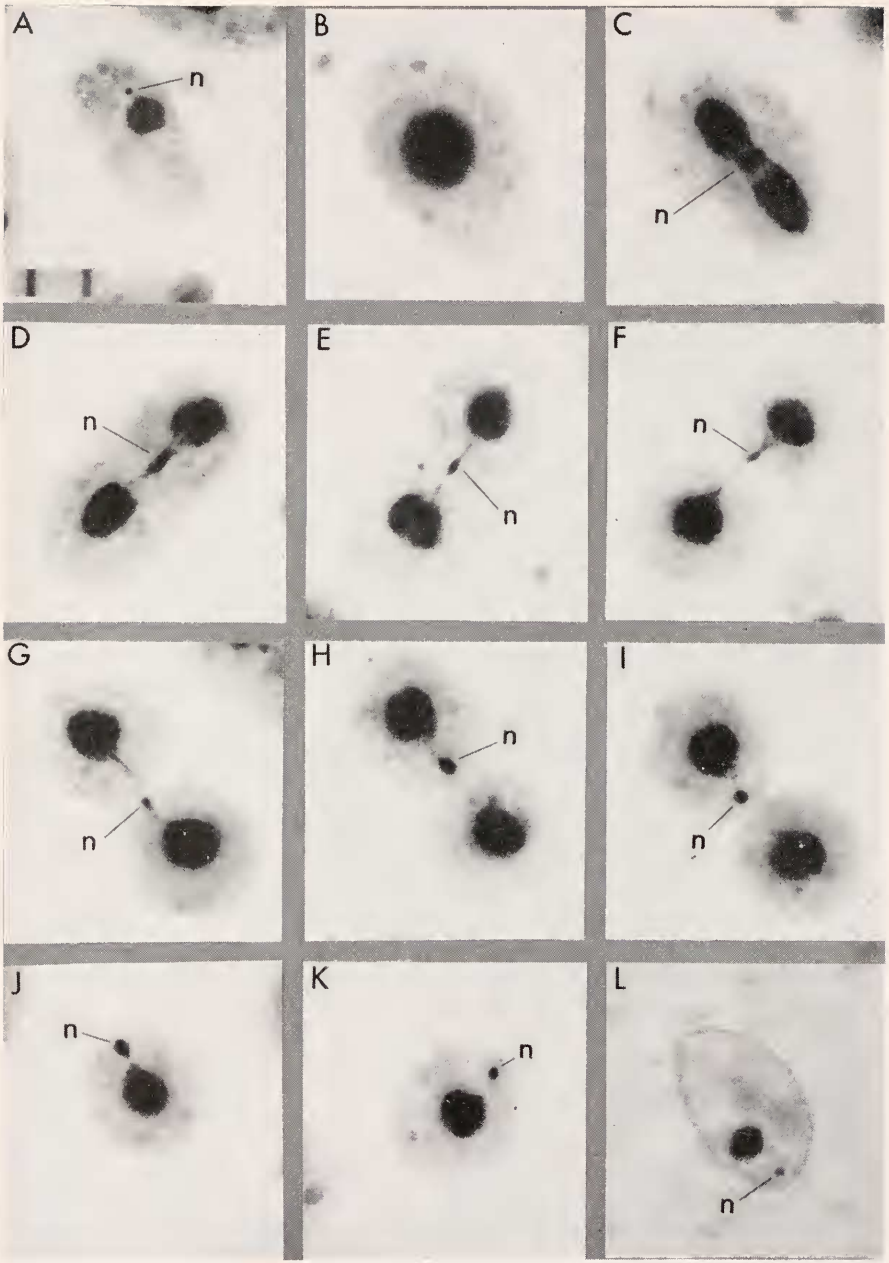

FIGURE 1. Photomicrographs of Feulgen-stained cells from various growth phases: Normal exponential multiplication (A), after temperature treatment (B), during and after the first synchronous division (C to K), and in maximum stationary phase (L). The distance between the two lines on the scale in (A) is 10 μ; "n" denotes a subnuclear aggregate (SNA). Further explanation in the text.

tion of synchrony the culture was exposed to seven temperature cycles. The temperature was changed every half hour between 28° and 33.9° C. One hour and 15 minutes after the end of the seventh cycle 80 to 85 per cent of the cells were in the visible stage of fission. The third sample was removed after 48 hours of subsequent growth when the cells were in the early stationary phase.

Figure 1 shows photomicrographs of cells in various stages of population growth and of the formation of the SNA's during division. In A the deeply stained SNA (n) is close to the macronucleus and resembles the micronucleus as shown by Holz, Scherbaum and Williams (1957) in mating type 1, variety 1, of *Tetrahymena pyriformis*. Figure 1, B shows a typical enlarged cell and nucleus after the end of the temperature treatment. No SNA from a previous division can be seen, although some were found in other preparations. In C the macronucleus elongates during the onset of synchronous division. A distinct portion of the nucleus seems to be "suspended" between the macronuclear halves pulling apart amitotically. In

TABLE I

Size and number of subnuclear aggregates (SNA's) in various growth phases

Sample No.	Number of cells with SNA (%)	Mean macronuclear volume in μ^3	Mean volume of SNA in μ^3	SNA/macronucl. volume ratio in %
1 Control exponent. multiplication	16	265.0	1.9	0.72
2 Prior to synchr. division	22	1063.0	x	x
3 After synchr. division	55	430.0	12.2	2.84
4 Max. station. phase	6	122.0	1.8	1.48

In order to determine the average percentage cells with SNA's, 100 cells of each type were examined. The macronuclear volume for each growth stage is the average for the 100 cells measured. The mean volume given for the SNA is the average of 50 measurements; "x" denotes that no measurements were made.

D to G this macronuclear remnant can be seen at various stages of cell division. In these phases of division the fragment still shows the typical granular composition of the macronucleus. However, somewhat later, when the fibrous connection between the macronucleus and the fragment disappears, the fragment tends to become spherical, the granular structure disappears, and the fragment becomes a dense homogeneous mass, resembling the micronucleus in this respect (I–L). Figure 1, J and K shows cells immediately after division. In cells of the early stationary phase of growth, SNA's were also found (L).

For a quantitative estimation of the size and number of the SNA's the experimental culture was sampled in various growth phases. The result is shown in Table I.

The number of cells with SNA's is relatively constant in exponentially growing cultures (16 per cent) and increases slightly in the course of the heat treatment.

However, after synchronous division SNA's were found in 55 per cent of the cells. On the assumption that the SNA's observed in the cells prior to division are carried through the synchronous division step, one can calculate that in approximately 45 per cent of the cells undergoing division new formation of SNA's took place.

The mean volume of the SNA's is relatively constant in the logarithmic phase and stationary phase of growth. It is approximately 2.0 μ^3. This value is 0.7 per cent and 1.5 per cent of the macronuclear volume at these two growth phases, respectively. After the synchronous division the average SNA volume is 12 μ^3, showing a six-fold increase as compared to normal values.

Evaluation of the Results and Discussion

In almost all cells examined only one SNA was found, but in some cases two or three SNA's could be observed in one cell. From the frequency with which

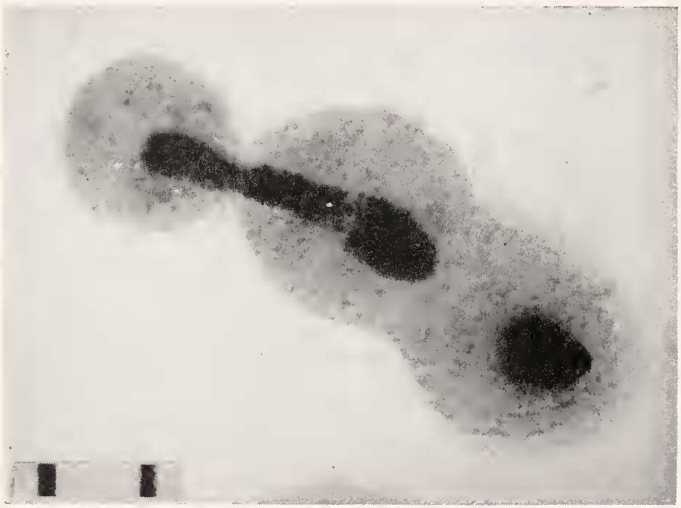

Figure 2. Photomicrograph of a Feulgen-stained cell during the first division after the heat treatment. The distance between the two lines on the scale is 10 μ. Further explanation in the text.

the SNA's occur at various growth phases it seems as if that they are broken down to Feulgen-negative material or are extruded from the cell. However, there is no evidence which might serve to evaluate either of these possibilities. The abnormally large nuclei of synchronized cells, together with the larger size of the SNA's of synchronized cells, might suggest that the size of the SNA's depends to some degree on the volume of the parent macronucleus. However, the size of the newly formed SNA's may vary, as can be seen in Figure 1, E–H. Furthermore, the size appears to be a function of the age of the SNA, since when first formed it is granular, similar to the macronucleus, and it then becomes homogeneous and smaller, apparently by condensation, before it disappears. In the present analysis we followed the formation of the SNA's with the Feulgen method for DNA only. However, nothing is known about the concentration of the basic proteins in these bodies. Basic proteins are normally found to be associated with

the DNA in the nucleus. A difference in stainability of the basic proteins in the micro- and macronucleus was observed by Alfert and Goldstein (1955) in mating types I and II of *Tetrahymena*. One could imagine that the original DNA basic protein ratio, as characteristic for the macronucleus and for the young SNA's in *Tetrahymena* GL, could change by preferential degradation of DNA in the course of the presumed condensation process occurring after formation of the SNA's.

Although the extrusion of macronuclear material in non-dividing cells and the formation of SNA's have been observed in various protozoan cells (see introduction), there is no conclusive evidence concerning the role which these phenomena play in the metabolism of the cells. Following the concept of strict equal distribution of parental DNA to the daughter cells one might be somewhat puzzled by this phenomenon. However, the high degree of polyploidy in the macronucleus suggests that such an equal distribution may not be a "*conditio sine qua non*."

A slight imbalance in timing of nuclear and cell division could cause this loss of DNA in the fission plane, and the failure for it to be incorporated into the daughter nuclei. In rare instances an "imbalance" of nuclear and cell division was observed in synchonized cells. For instance, Figure 2 shows a cell during synchronous division, dividing into three instead of two daughters. In the right part of the cell, nuclear division is completed, while cellular division lags slightly behind. In the left part of the cell the macronucleus is in division, while cytoplasmic division is far more advanced than in a normal cell with a comparable nuclear figure. That such irregularities hardly affect the viability of the cells is not surprising in view of the fact that the protozoan macronucleus is a highly polyploid system. Sonneborn (1947) concludes, from genetic evidence, that the macronucleus of *Paramecium aurelia* must contain about 40 diploid "subnuclei." These observations suggest the interesting problem of to what extent this high polyploidy of the macronucleus could be reduced experimentally. For instance, in starving cultures of *Tetrahymena pyriformis* strain S, Weis (1954) found a reduction in cell size to less than 10 per cent of the normal volume. These cells "regulated" back to their normal size upon addition of nutrients to the culture medium. If one assumes an almost constant nucleo/cytoplasmic ratio and 40 diploid "subnuclei" (as found for *Paramecium*), one might expect the starved cells to carry only 4 diploid "subnuclei."

Opposed to the view that the loss of subnuclei during binary fission is an arbitrary phenomenon, based on mere chance, is the idea which attributes a strict regulatory function to these processes. Findings by Kidder and Claff (1938) seem to substantiate this point of view. These authors investigated the life cycle of *Colpoda cucullus* and described chromatin extrusion following each division in regular and predictable fashion. This "budding" of the macronuclei occurs almost synchronously in the two daughter cells. In contrast to the loss of DNA *during* the fission process, as described for synchronized cells of *Tetrahymena*, we have in *Colpoda cucullus* an example of active regulation or reorganization of some sort *after* the daughter cells are formed.

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Summary

1. The formation of "subnuclear aggregates" (SNA's) is studied quantitatively in synchronously-dividing cells of *Tetrahymena pyriformis* strain GL.

2. In normal cultures approximately 16 per cent of the cells were found to contain SNA's. This value rises to 55 per cent after synchronous division. The SNA/macronuclear volume ratio is 0.72 per cent in normal cells and 2.8 per cent in cells after synchronous division.

3. The possible significance of the formation of SNA is discussed.

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